Evaluation of Phytochemicals, antioxidant and antimicrobial activity of *in vitro* culture of *Vigna unguiculata* L. Walp.

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Abstract: Tissue culture of *V. unguiculata* L. Walp. was done on MS medium supplemented with different concentrations of phytohormones. Maximum callusing was observed in basal MS medium containing KN (5 ppm) and NAA (1 ppm) and minimum callusing in MS + BAP (1ppm) + IAA (0.1 ppm). The total phenolic content and total flavonoid content was found to be more in callus derived from MS + KN (5 ppm) and NAA (1 ppm) as compared to *in vitro* grown seedlings. The ethyl acetate fraction of callus was evaluated for its antimicrobial and antioxidant activity.

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1. Introduction

During last two decades there has been tremendous rise in infectious diseases which has led to the death of several individuals. This has been attributed to increased resistance of pathogenic microbes to antimicrobial agents/drugs and indiscriminate use of synthetic antibiotics. Thus, there is continued search for new antimicrobials especially of natural origin.

Medicinal plants possess myriads of bioactive compounds having therapeutic potential (Sharma *et al*, 2009; Bhatia *et al,* 2008a; Bhatia *et al*, 2008b). These plants have been used traditionally as antimicrobials. There are reports on antimicrobial activity of plants (Mathur, 2007; Alam *et al*, 2011). This has provided an impetus to explore more of these plants viewing the burgeoning demand for antibiotics. As a result of overuse of plants there is considerable decrease in their number in most parts of the world.

The present study is in continuation of author's previous study (Vats, 2012) on *Vigna unguiculata* (L.) Walp. (Common name: Cowpea) exploring the significance of Plant Tissue Culture as an alternative source of plant secondary metabolites together with their efficacy. *V. uguiculata* seeds form a cheap source of protein in developing countries. Cowpea has been used traditionally against fever (Hutchings *et al*., 1996), boils (Duke, 1990), stomach worms (Chopra *et al*., 1986), urinary problems (Kritzinger *et al*., 2004) and others.

The present work was undertaken to evaluate the phytochemical constituents of *V. unguiculata* callus culture and their efficacy as antioxidant and antimicrobial agent.

2. Material and Methods

The seeds of *V. unguiculata* were surface sterilized with 0.1% $HgCl_2$ (Mercuric chloride) in laminar flow hood and washed with sterile distilled water (Vats, 2012). Epicotyl was used as the explant from *in vitro* grown seedling. The explants were then inoculated in MS media supplemented with various concentrations of auxins viz., 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and NAA (Naphthalene Acetic Acid) and IAA (Indole Acetic Acid) and cytokinins KN (Kinetin) and BAP (Benzylaminopurine). All cultures were maintained at 25±2ºC under light intensity (1200 lux) for photoperiod of 16h light and 70±10 relative humidity. The cultures were maintained for about 6 months by periodic subculturing of 6-8 weeks time interval.

2.1 Extraction

The *in vitro* grown seedlings and callus tissue (10g, each) were dried at 50°C till constant weight was achieved. It was then powdered and extracted with 80% methanol (100ml) in orbital shaker at 120rpm for 24 hr. Methanolic fraction was re-extracted with ethyl acetate (EA) and used for further analysis.

2.2 Total phenolic content (TPC)

TPC was measured according to the Folin-Ciocalteau method (Vats, 2012). Briefly, 0.5 mL of distilled water and 0.125 mL of the sample was added to a test tube. Folin-Ciocalteau reagent, sodium carbonate solution and distilled water was added successively and allowed to stand for 90 minutes. The absorbance was measured at 760nm. Total

phenol content was expressed as gallic acid equivalents (GAE) in (mg GAE/gdw) dry material.

2.3 Total Flavonoid content (TFC)

Total flavonoid content was estimated using the method of Chang *et al* (2002) and expressed as quercetin equivalent (mg/gdw). Quercetin was used to make the calibration curve. Extracts (0.5 mL) were mixed with 95% ethanol (1.5 mL), 10% aluminum chloride (0.1 mL), 1M potassium acetate (0.1 mL) and distilled water (2.8 mL). After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm.

2.4 DPPH free radical scavenging activity

Sample extract (1ml) was mixed with 0.3mM DPPH reagent (1ml) and allowed to stand at room temperature for 30 minutes in dark. The absorbance was taken at 517nm. Radical scavenging activity was calculated as IC_{50} value (Vats, 2012).

2.5 FRAP assay

Acetate buffer (300mmol/l), 10 mmol/l 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mmol/l HCl and 20 mmol/l $FeCl_3$ x $6H_2O$ in distilled water was prepared. 25ml of acetate buffer, 2.5ml TPTZ solution and 2.5ml $FeCl_3$ x $6H_20$ solution was mixed to make working solution. 50µl of sample extract was mixed with 1.5ml of FRAP reagent and monitored up to 5 min at 593nm. Absorbance was compared with calibration curve of aqueous solution of known Fe (II) concentration (µM/l) (Vats, 2012).

2.6 Sources and maintenance of organisms

Gram-positive organisms (*Staphylococcus aureus* (MTCC 96)*, Proteus mirabilis* (MTCC 425), *Bacillus cereus* (MTCC 430), *Bacillus subtilis* (MTCC 121) and Gram-Negative organisms (*Pseudomonas aeruginosa* (MTCC 429)*, Escherichia coli* (MTCC 443) were maintained on Mueller-Hinton Agar medium (MHAM).

Antimicrobial Bioassay

Microbial suspension was prepared in sterile normal saline and adjusted to 0.5 Macfarland standard (10^8 Cfu/ml). Test organisms were inoculated uniformly on labeled plates. Wells were made using sterile cork borer (5mm diameter) and poured with 100 µl (1000 µg/ml) of extract. Ethyl acetate was used as negative control. The plates were incubated at 37°C for 24 hours. Antimicrobial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation. Ciprofloxacin (0.05%) was used as positive control (NCCLS, 2000).

2.7 Determination of Minimum Inhibitory Concentration (MIC)

To measure the MIC values, stock of 1500 µg/ml extract was serially diluted to 25, 50, 100, 200, 400, 800, 1000 µg/ml (NCCLS, 2000) and mixed with equal volume of MHAM. A loopfull of microbial suspension (10^8 Cfu/ml) was inoculated in different test tubes. The tubes were incubated at 37°C for 24-48 h. Two control tubes, tube containing the growth medium, saline and the inoculum, were also prepared. The lowest concentration of the extract that produced no visible microbial growth when compared with the control tubes was regarded as MIC.

2.8 Determination of Minimum Bactericidal Concentration (MBC)

MHAM plates were categorized into different sections and labeled with the different concentrations on the base of the plates. The contents of MIC tubes were plated in the respective sections of the plates. The plates were incubated for 18-24 hr at 37 °C. The highest dilution that yielded no single bacterial colony was taken as the Minimum bactericidal Concentration (Akinyemi *et al,* 2005).

2.9 Statistical analysis

The experiments were repeated five times and the results were expressed as mean ± standard deviation.

3. Results and discussion

Basal MS medium was used for *in vitro* germination of the seeds of *V. unguiculata*. Germination started after 2-3 days (Fig. 1). Epicotyl was used as explant from *in vitro* grown seedling. Callusing started from the cult ends of explant. The callus was fragile and light brown in colour. Maximum induction of explant (88%) and callusing was observed in basal MS medium supplemented with 5 ppm KN and 1ppm NAA (Fig. 2). The results were almost at par using hypocotyl explant in previous study on *V. unguiculata* (Vats, 2012). There are other reports on callus induction using higher cytokinin concentration (Diallo *et al,* 2008**)**. Good callusing was observed in three combinations of hormones viz., 2, 4-D (1ppm); 2, 4-D (1ppm) + KN (0.01ppm) and BAP (1.5ppm) + KN (1.5ppm). Minimum induction of explant and callusing was observed in MS + BAP (1 ppm) + IAA (0.01ppm). Other combination of hormones showed moderate callusing (Table-1). The media in which maximum amount of callusing was observed were sub-cultured periodically after 6 weeks and maintained further and analysis (Figure 1). Callus in Cowpea was observed in MS medium supplemented with 1 µM NAA and BAP (Odutayo *et al*, 2005). Amitha and Reddy (1996) reported smooth nodular callus from zygotic embryos.

Table 1: Callusing in different concentrations of phyto-hormone using epicotyl explants

2,4-D	NAA	KN	BAP	IAA	Response (%)	Callusing
1	-	-	-	-	69 ± 1.5	+++
-	1	5	-	-	88 ± 1.8	++++
1	-	0.1	-	-	62 ± 1.5	+++
-	-	1.5	1.5	-	65 ± 0.8	+++
-	-	-	5	-	57 ± 0.5	++
-	-	-	1	0.1	45 ± 1.1	+
-	0.5	2.5	-	-	54 ± 1.2	++

Fig. 1: *In vitro* grown seedling of *V. unguiculata*

Fig. 2: Callus induction in MS + KN (5ppm) + NAA (1ppm)

Phenolic compounds are diverse group of metabolites found in plants. These bioactive compounds are known to possess antioxidant/free radical scavenging activity.

Flavonoids, one of the polyphenolic compounds, possess therapeutic value and used in treatment of various diseases.

Values are Mean ± S.D; (++++ = very good; +++ = good; ++ = moderate; + = poor)

In the present investigation a linear calibration curve of Gallic acid was obtained (R^2 =0.98). TPC of *in vitro* grown seedling and callus was found to be 68 ± 0.86 and 74 ± 0.58 mg/g GAE, respectively. In an earlier study TPC of hypocotyl derived callus from same media composition was evaluated to be 60 ± 0.64 mg/g GAE (Vats, 2012). This suggests the differential response of various explants in terms of metabolite content, which might depend on the physiology and metabolism of explant and callus derived from it.

A linear calibration curve of quercetin was obtained (R^2 =0.99). TFC in *in vitro* grown seedling was found to be 1.10 mg/gdw. Callus showed more TFC (1.60 mg/gdw) as compared to *in vitro* grown seedling. Synthesis of secondary metabolites is dynamic process. These bioactive compounds are synthesized in specialized tissues and are also further metabolized. In the present study the biosynthetic pathways leading to the formation of flavonoids might be more active or the flavonoids might not have been channelized towards degradative pathway, which led to enhanced recovery of flavonoids in callus tissue.

The callus tissue was assessed for its antioxidant potential. The IC_{50} value of seedling was found to be 90 ± 1.4 µg/ml. Callus showed comparatively better activity with IC_{50} value 70 ± 1.1 µg/ml. the FRAP assay revealed similar results wherein the reducing activity was observed to be more in callus (870 ± 2.5 µM/l) as compared to *in vitro* grown seedling (850 ± 1.9 µM/l). Hypocotyl derived callus from same media composition was observed to have lower antioxidant activity (Vats, 2012). Phenolics have been known to possess antioxidant potential because of the presence of –OH group. In the present study callus showed better antioxidant activity, this might be due to increased production of flavonoids and phenolics.

The EA extract of callus (500µg/ml) showed maximum activity in terms of IZ against *E. coli* (16 ± 0.5mm). Minimum antibacterial activity was shown against *P. aerugenosa* (IZ-11 ± 0.8mm). The antibacterial activity was in the range of 12-14mm in other organisms tested (Fig. 3). Mathur *et al* (2007) reported antimicrobial flavonoid fraction of few medicinal plants.

Maximum antimicrobial activity of Ciprofloxacin (0.05%) was observed against *B. subtilis* and minimum activity in *S. aureus*. The activity was at par against *P. mirabilis* and *P. aerugenosa*.

MIC for *E. coli* was found to be 110 ± 1.2 µg/ml followed by *B. cereus* and *S. aureus* (125 ± 0.9 and 138 ± 1.0 µg/ml, respectively). MIC for *B.*

subtilis and *P. aerugenosa* was evaluated to be 150 and 168 µg/ml, respectively. MBC was found to be at par with MIC in *E. coli*, *S. aureus* and *B. cereus*. In *B. subtilis*, *P. mirabilis* and *P. aerugenosa* the MBC (160 ± 1.5, 155 ± 0.5 and 175 ± 0.6 µg/ml, respectively; Fig. 4) was evaluated to be more than MIC.

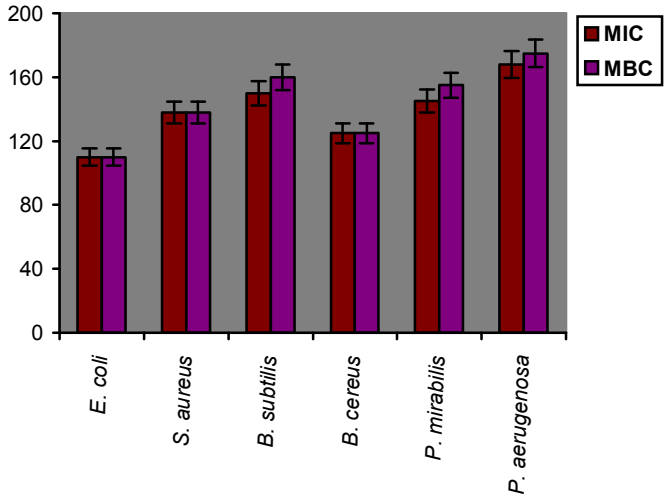

Fig. 3: Antimicrobial activity of EA extract (500µg/ml) of callus tissue *V. unguiculata*

The antibacterial activity of extract might be due to phenolic and flavonoid content of callus. The differential activity may be due to the presence of various phyto-compounds in varying concentrations and adversely affecting the metabolism of microbes.

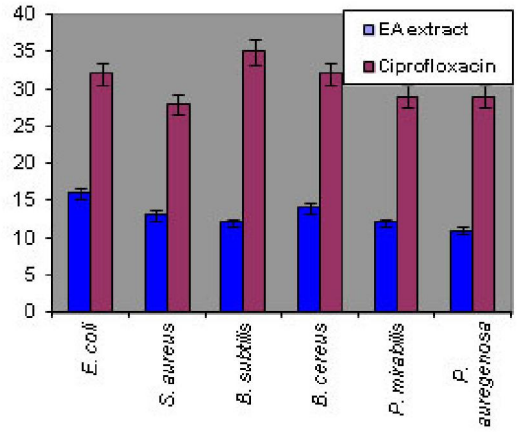

Fig. 4: MIC and MBC (µg/ml) of EA extract of callus of *V. unguiculata.*

Conclusion

The present study concludes that various explants respond differentially in terms of metabolite content on same hormonal combinations in tissue culture media. It also confirms callus culture of *V. unguiculata* to be a potential source of flavonoids and phenolics, and an antimicrobial agent.

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